

## Understanding why fat, oil and grease (FOG) bioremediation can be unsuccessful

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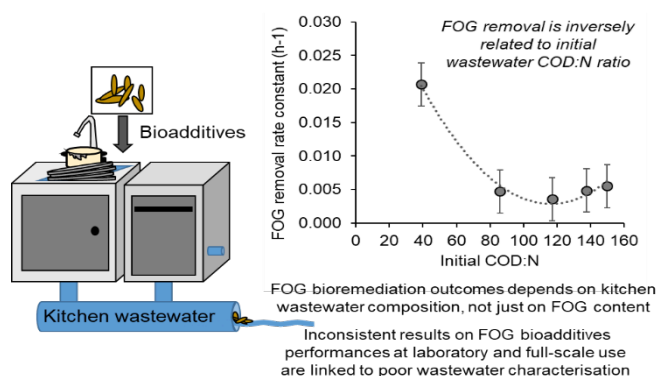
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### Highlights

- FOG biodegradation rates varied with wastewater composition between 5 - 26 mg/l/h.
- FOG removal decreased from 24 to 10 mg/l/h when glucose was substitute for starch.
- FOG removal was inversely related to initial COD:N.
- FOG removal started in the stationary phase of bacterial growth in all wastewaters.

### Graphical Abstract



### Abstract

Commercial kitchen wastewaters are typically strong organic and fat-rich effluents, often identified as major contributors to fatberg formation and associated blockages in sewers. Experimental trials were done using synthetic kitchen wastewater to understand the complex reactions involved in microbial remediation in grease traps/separators prior discharge in sewers. The principle organic components (FOG, carbohydrate and protein nitrogen), were varied using ranges observed in a previous study on real kitchen wastewater characterisation. A model bacterium, *Bacillus licheniformis* NCIMB 9375, was used to evaluate microbial utilisation of the different organic fractions in relation to fat, oil and grease (FOG) degradation. Novel results in the treatment of these effluents showed that, the presence and concentration of alternative carbon sources and the ratio of carbon to nitrogen (COD:N) had great influence on FOG-degradation response. For example, FOG removal decreased from 24 to 10 mg/l/h when glucose was substitute for starch at equivalent concentrations (500mg/l); and from 26 to 5 mg/l/h when initial COD:N increased from 45:1 to 147:1. The dominant influence of COD:N was validated using a commercial bioadditive and real kitchen wastewater adjusted to different COD:N ratios, confirming the strong influence of kitchen wastewater composition on bioremediation outcomes. These results can therefore have major implications for biological management of FOG in kitchens and sewers as they provide a scientific explanation for bioremediation success or failure.

**Keywords:** bioadditives; bioadditions; kitchen wastewater; fatberg; lipids; FOG

# 1 Introduction

A wide variety of industrial, commercial and domestic establishments produce wastewater rich in fats, oils and greases (FOGs) which can form deposits in sewer pipes and contribute to the formation of sewers' fatbergs (Brooksbank *et al.*, 2007; Montefrio *et al.*, 2010). Alongside physical processes (i.e. grease separators), these FOG discharges can be managed with biological products (or bioadditions), which can be added to a grease separator/trap or to kitchen drains to enhance the sewers' natural bioremediation ability. Over the years, lab-based or full-scale addition of oil-degraders to FOG-rich wastewater has produced variable results (Mendoza-Espinosa and Stephenson, 1996; Brooksbank *et al.*, 1997, Wakelin and Forster, 1997). Failure cases have always been associated with poor microbial adaptation to the environment, inadequate dispersion or the presence of inhibitors or toxic compounds (Watanabe *et al.*, 2000). Notwithstanding the positive outcome of many bioaddition treatments, there is still very limited information on the science underpinning the process and, in particular on the impact of the physicochemical composition of the wastewater to treat and on how the different components could influence the bioremediation process.

For example, the degradation of triglycerides, the dominant component of FOG in kitchen wastewater (KWW), starts with a hydrolytic step mediated by lipolytic enzymes or lipases. Lipases production, can be enhanced (Hasan *et al.*, 2006), reduced or completely inhibited by particular carbohydrates and organic nitrogen sources and concentration (Eltaweel *et al.* 2005; Ertuğrul *et al.* 2007; Takaç & Marul 2008). Rathi *et al.* (2001) found optimum lipolytic activity from *Burkholderia cepacia* at 1% glucose over a test range of 0.25 – 2.5% in the presence of 1.5% oil. El-Shafei and Rezkallah (1997) reported changes in lipase activity from two *Bacillus* spp. when fodder yeast medium was supplemented with glucose. In particular, lipase production was completely inhibited at the low and high concentrations with an optimum at 0.6%. Besides these responses, lipase production does not necessarily lead to microbial removal of hydrolysed free fatty acids. In a mixed bacterial culture, Tzirita and Quilty (2009) determined that

*Bacillus* species were responsible for lipase production, but only non-lipolytic *Pseudomonas putida* was capable of assimilating the free fatty acids.

Another important parameter for an effective biological treatment is the carbon to nitrogen balance in the wastewater, expressed as COD:N ratio. Microorganisms utilise carbon substrates through oxidation as a source of energy (catabolism) or, together with nitrogen, for growth and protein formation (anabolism). Under balanced nutritional conditions catabolism and anabolism are tightly coupled such that the amount of energy generated by carbon oxidation is dominantly used to fuel growth, leading to cell growth yields near maximum theoretical values. Typically a COD:N ratio of 20:1 is recommended for efficient treatment of WW of domestic composition (Grady *et al.*, 2011). This ratio provides enough carbon to allow all available nitrogen to be co-synthesised into new biomass together with just the right amount of carbon energy to drive the process. Gurd *et al.* (2019) reported ratios of 60:1 and 50:1 for sink and mixed effluents wastewater and 75:1 for dishwasher discharges, suggesting a significant N deficiency for successful biological treatment leaving excess organic carbon undegraded. Nevertheless, microbial populations have the ability to adjust their metabolic processes in response to nutrient deficiency/carbon excess, whereby the balance between synthesis and energy shifts towards surplus energy production under carbon excess conditions (Dauner *et al.*, 2001). Thus, organic matter in WW with COD:N values greater than 20:1, such as that discharged by commercial kitchens, can potentially be fully degraded without additional nitrogen but at reduced cellular yields. Jefferson *et al.* (2001), showed that an appropriate C:N balance in greywater biological treatment was able to eliminate the macronutrient-limiting conditions often hindering substrate degradation, such as in the case of fat in kitchen wastewater. Many lab-based FOG degradation studies, have used FOG as the sole carbon source (Becker *et al.* 1999; Loperena *et al.* 2006); or arbitrary concentrations of other substrates - either higher (Tano-Debrah *et al.*, 1999), or in atypical proportions (Brooksbank *et al.*, 2007; Tzirita and Quilty, 2012) to real wastewater conditions.

FOG management in food service establishment (FSE) kitchen wastewater is a more complex process than just lipid degradation. Most studies investigating

multi-substrate systems, have only evaluated the impact of the presence of alternative carbon sources rather than co-monitoring their microbial uptake. The work aims to close these gaps by delivering a more thorough evaluation of bacterial response to realistic variations of each kitchen wastewater component.

## **2 Materials and methods**

Chemicals were obtained from Fisher Scientific, UK unless otherwise stated.

### **2.1 Bacterial inoculum and growth monitoring**

*Bacillus licheniformis* NCIMB 9375 was purchased from the UK national culture collection (NCIMB), selected amongst others for its high lipid-degradation ability (Voigt et al., 2007). Stock cultures were maintained on nutrient agar (Oxoid CM0003,) at 4°C and sub-cultured monthly. Bacterial growth was monitored by spread plate technique on Oxoid nutrient agar.. Cell dry weight (CDW) was determined for the glucose-only batch reactors according to standard methods. The FOG-remediation commercial bioadditive was a bacterial multi-species liquid preparation with a 0.1-1% nominal active microbial culture.

### **2.2 Degradation trials**

Batches of 16-20 1 l Erlenmeyer flasks containing 500 ml of culture media (synthetic or real wastewater) were used to monitor each condition. Flasks were dosed with 1 ml of bacterial inoculum to achieve an in-situ dose of  $5 \cdot 10^5$  cfu/ml. *Bacillus* inocula were pre-cultured in nutrient broth to  $2.5 \cdot 10^8$  cfu/ml, confirmed by spread plate counting. The cultures were then incubated at 40°C and 150 rpm (SciQuip Incu-Shake TL6-5R, SciQuip UK) for up to 62 hours. Triplicate sacrificial samples were removed at 8, 16, 38, and 62 hours and analysed immediately for cell counts and FOG content. Further subsamples were stored at -18°C for carbohydrate and TN analysis. Sterile controls were treated in the same manner without inoculation.

In order to directly compare removal of the different organic WW components, concentrations, removal rates, and bacterial growth yields were

normalised to theoretical COD equivalents using conversion factors of 2.88, 1.07, 1.5 and 1.42 for FOG, carbohydrate, protein and biomass respectively, according to Grady *et al.* (2011).

### 2.2.1 Synthetic wastewater

A basal mineral medium was designed using a kitchen wastewater characterisation previously reported (Gurd *et al.* 2019). The synthetic KWW composition (recipe reported in table 1S) contained calcium chloride dihydrate (100 mg/l), potassium chloride (70 mg/l), magnesium sulphate heptahydrate (40 mg/l), ammonium chloride (10 mg/l), iron chloride (0.27 mg/l), zinc sulphate heptahydrate (0.16 mg/l), copper sulphate (0.07 mg/l), manganese sulphate monohydrate (0.045 mg/l), cobalt nitrate hexahydrate (0.002 mg/l) and ammonium heptamolybdate tetrahydrate (0.001 mg/l), together with a non-ionic surfactant Triton X-100 (14 mg/l). Rapeseed oil was added directly as FOG (150-800 mg/l). Solutions of glucose, and/or cornflour (Tesco, UK) were added at varying concentrations (100, 500, 1000, 2000, 4000 mg/l) as a source of carbohydrate, whereas soy protein acid hydrolysate (SPH, Amisoy) was added mainly as nitrogen source (the additional carbon carried by the hydrolysate was also considered in the calculations). SPH concentrations were varied (150, 300, 450, and 600 mg/l), to achieve initial TN concentrations of 20, 40, 60 and 80 mg/l, with associated amino acid concentrations of 124, 248, 373 and 497 mg/l, calculated as 82.8 wt% from the manufacturers' information datasheet (Sheffield Bioscience, 2014). Four trials were conducted wherein one substrate concentration was varied at a time ie. FOG, glucose, starch or organic nitrogen, whilst keeping the other parameters constant.

Flasks were autoclaved at 121°C for 20 minutes. Media was buffered to pH 7 by addition of 30 ml/l of a sterile 1M sodium phosphate buffer after autoclaving to prevent precipitation of calcium phosphate. 30 mg/l of the anionic surfactant sodium dodecyl sulphate, at a similar concentration to that measured in real wastewater was also added post-autoclave to prevent thermal degradation. Filter-sterilised rapeseed vegetable oil (Tesco, UK) was added dropwise to the flasks with a sterile pipette. Concentrations of the organic components were

varied to achieve the test parameters reported in Table 1 covering ranges previously determined in real KWW (Gurd *et al.* 2019).

Following media preparation, flasks were shaken overnight to emulsify the oil before bacterial dosing.

**Table 1.** Test conditions for the four experimental trials showing concentrations of individual substrates and COD(theoretical):N ratios, compared to concentration ranges in real kitchen wastewater (Gurd *et al.* 2019).

Organic substrate range in real KWW (mg/l)	Organic component varied in the test	Test concentrations (mg/l) and COD:N ratios	Constant parameters (mg/l)
Total FOG: 200 – 2000	Rapeseed oil (as a model for FOG)	150, 300, 450, 600, 800 45, 53, 61, 69, 80	Glucose 1200 Starch 220 SPH 400 (TN 52)
Total carbohydrate (simple sugar): 100 - 4000	Glucose (soluble monosaccharide)	100, 500, 1000, 2000, 4000 35, 42, 53, 73, 112	Oil 580 Starch 0 SPH 400 (TN 52)
Total carbohydrate (complex): 100 - 4000	Corn starch (particulate carbohydrate)	100, 500, 1000, 2000, 4000 33, 44, 53, 75, 111	Oil 580 Glucose 0 SPH 400 (TN 52)
TN: 24 - 80	Soy protein hydrolysate, SPH, (as total nitrogen)	150, 300, 450, and 600 (TN 20, 40, 60, 80) 45, 56, 80, 147	Oil 580 Glucose 500 Starch 500

### 2.2.2 Real wastewater

Kitchen wastewater was collected from a food service establishment on Cranfield University campus. The samples represented water entering the sewers from the pot-wash sink, with a capacity of about 40 l. The sampling schedule aimed at capturing effluents from a restaurant at its peak time after the lunchtime service. Sample containers were shaken vigorously to emulsify and homogenise the free FOG fraction. Subsamples of 500 or 250 ml were transferred into conical shake flasks and inoculated with either *B. licheniformis* or a commercial bioadditive product at a dose of  $5 \cdot 10^5$  cfu/ml and cultured as in the synthetic WW trials.

COD:N ratio in the wastewater was varied by addition of starch or ammonium chloride. Composition of the different batches used are detailed in Table 2S in Supplementary Material.

### **2.3 FOG, proteins and carbohydrates quantification**

FOG (oil) concentration was quantified using the modified Gerber method described in Gurd *et al.* (2018). Briefly, 200 ml samples were weighed into transparent polycarbonate centrifuge bottles (250 ml Nalgene, Fisher UK). 1 ml of 10% sodium caseinate solution was added to the emulsified sample, with hydrochloric acid to lower the pH until precipitated particle aggregation is visually confirmed, based on a 30 second mixing period. The sample is placed horizontally on a rotary shaker at 90 rpm for 15 minutes or until the aqueous phase is clear and then centrifuged at 2000g for 10 minutes (Sorvall Legend RT+, DJB labs, Newport Pagnell UK). The supernatant is discarded and 1% sodium hydroxide added to the precipitate and residual fluid to raise the pH to 7 to partially re-dissolve the casein generating a slurry for easy transfer.

The method then follows the Gerber method (BS ISO 2446:2008) with some modifications. The slurry is layered onto 10 ml of sulphuric acid in a 0-1% butyrometer (Funke Gerber, VWR, UK), followed by a few ml of DI water that were used to rinse down the walls of the centrifuge bottle. 1 ml of isoamyl alcohol is added and the total volume topped up with distilled water. An acid resistant bung is inserted and the butyrometer shaken vigorously for 90 seconds. The butyrometer is centrifuged in a heater Gerber unit (Funke Gerber Nova Safety, VWR, UK) for 10 minutes followed by tempering in a water bath at 65°C for 3 -10 minutes. An average specific gravity of 0.89 g/ml was used for all samples. Samples harvested after 16 hours were pre-centrifuged at 10000g for 20 minutes to remove bacterial biomass before the supernatant was decanted into a new centrifuge bottle for the precipitation/flocculation stage of the quantification method. FOG concentrations were converted to COD equivalents by multiplying by a factor of 2.88 (Grady *et al.* 2011).

Nitrogen uptake was monitored as soluble total nitrogen (TN) in samples filtered through a 0.45 µm syringe filter, using a Shimadzu TOC-V analyser with a total

nitrogen module (TNM-1). Equivalent soluble protein was calculated by multiplying by the generic nitrogen conversion factor of 6.25 for real wastewater, and specifically 6.37 in synthetic wastewater determined from the total amino acid/TN ratio for Amisoy SPH (82.8/13.0) (Sheffield Bioscience, 2014). Glucose and starch concentrations were quantified spectrophotometrically by the Dubois method (Dubois *et al.*, 1956) with a Jenway 6300 Visible spectrophotometer at 490 nm, with the soluble fraction filtered through a 0.45  $\mu$ m syringe filter. Concentrations for protein and carbohydrate were converted to COD equivalents by multiplying by 1.5 and 1.07, respectively (Grady *et al.* 2011). There was no obvious impact on carbs concentration measurements when either FOG or protein concentrations were varied, i.e. there was variability between samples but no trend with concentration. Initial measured concentrations correlated well with predicted concentrations based on recipe.

## 2.4 Data analysis

All measurements were conducted in triplicate, unless explicitly reported. Apart from the glucose variation trial where one batch failed, data are presented as means of triplicate independent experiments with the errors estimated in terms of the standard deviation. All numerical and statistical analyses were performed in Microsoft Excel.

## 3 Results and discussion

The impact of different bacterial dosing levels ( $10^4 - 10^7$  cfu/ml) on FOG removal was tested using the reference bacteria *B. licheniformis* NCIMB 9375 and a commercial multi-strain bioadditive product. Bioadditive products are typically dosed at  $10^4 - 10^5$  cfu/ml in commercial applications, while academic studies have employed higher starting doses,  $10^5 - 10^7$  cfu/ml (Loperena *et al.*, 2009, Tzirita and Quilty 2009).

Both *B. licheniformis* and the commercial product did not show significant differences on maximum population density after 60 hrs or FOG removal (Supplementary data. Figure 2S).



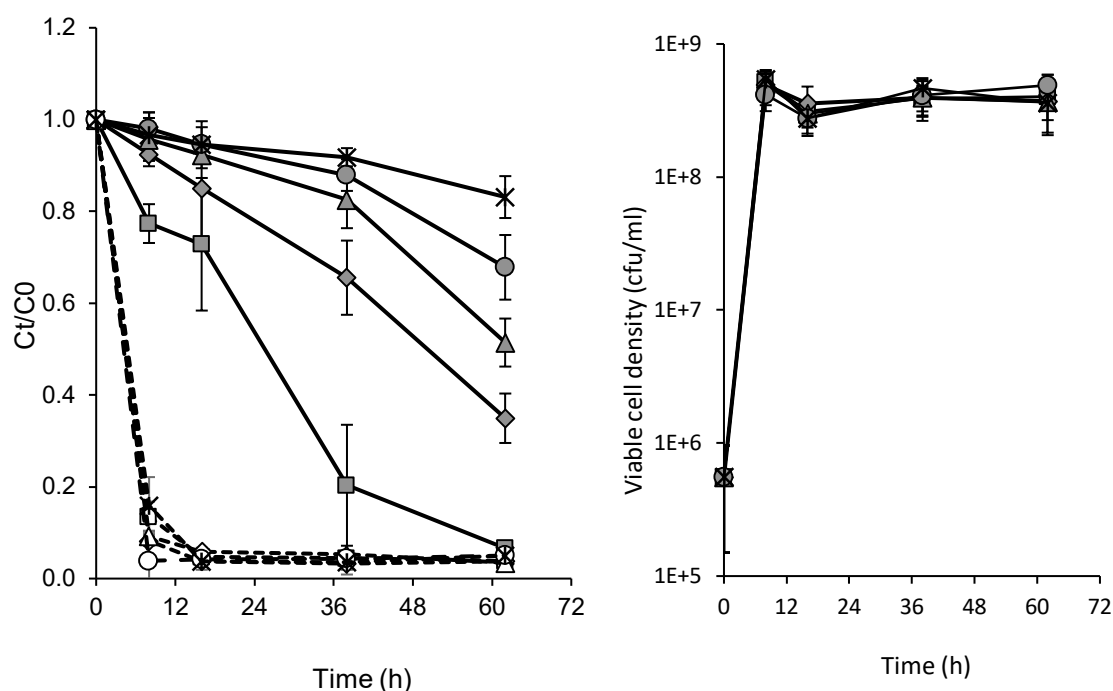
### 3.1 Influence of different initial FOG concentration

FOG removal rates were measured in batch tests containing different initial oil concentration (150, 300, 450, 600 and 800 mg/l) which reflected the range observed in real KWW (Gurd *et al.* 2019). Log phase bacterial growth was observed during the first 8 hours in association with rapid depletion of soluble carbohydrates, whilst, except for the lower FOG concentration (150 mg/l), minimal FOG removal was observed (Figure 1A), compared to  $95 \pm 4.4\%$  extraction efficiency in control samples. Bacterial populations fell by approximately half from 8 to 16 hours before recovering as a reflection of diauxic growth as they switched from metabolising carbohydrates to FOG. This growth-related response is consistent with previous lipolytic activity studies in multi-substrate media (Castro-Ochoa *et al.*, 2005; El-Shafei and Rezkallah, 1997; Handelsman and Shoham, 1994; Sharma *et al.*, 2014) suggesting a bacterial preference for co-substrates to support growth followed by a switch to FOG utilisation during stationary phase. Das *et al.*, (2010) determined that *B. coagulans* RK-02 produced maximum lipase activity after exhaustion of glucose, which had supported log phase growth. The FOG removal data were fitted to two common enzyme kinetic models: the Michaelis Menten model and the Andrews substrate inhibition model (Supporting Material, Figure S1), and displayed a better fit for the Andrews substrate inhibition model. This yielded kinetic constants of  $V_{\max}$  29 mg/l\*h;  $K_s$  524 mg/l;  $K_i$  39 mg/l and a critical fat concentration to inhibition (when  $v = V_{\max}$ ) of 143 mg/l.

This degree of substrate inhibition is considerably higher than previously determined. For instance, Loperena *et al.* (2006) calculated a critical fat concentration of 360 mg/l for substrate inhibition to growth, over a range of initial fat concentrations from 200 – 1000 mg/l. Whereas, Jung *et al.* (2002) reported a marked drop in COD removal from dairy wastewater by activated sludge from 86%, 75% to 0% with FOG supplementation of 400, 600 and 800 mg/l respectively.

(A)

(B)



**Figure 1.** (A) Time course of oil removal (solid lines) and soluble carbohydrates (dashed lines), and (B) bacterial growth, for *B. licheniformis* 9375 dosed at  $5E+5$  cfu/ml using synthetic wastewater with different initial oil concentration (■ 150, ◆ 300, ▲ 450, ● 600 and \* 800 mg/l).

These initial results suggest the need for additional experiments to investigate the influence of the different organic fractions on FOG removal to provide an understanding of the bacterial preference for each co-substrate in relation to FOG.

### 3.2 Influence of carbohydrates on FOG removal

The rapid reduction in soluble carbohydrate, prior to the initiation of FOG removal in the experiment reported in 3.1, suggested that glucose and FOG were metabolised sequentially. Glucose is a monosaccharide and thus up-taken into the bacterial cell directly, whilst starch and triglyceride FOG molecules must first be hydrolysed by extracellular enzymes. To investigate this further, culture media were prepared with either glucose or corn starch as a source of carbohydrate. Initial concentrations of 100, 500, 1000, 2000, 4000 mg/l were used to reflect the range observed in real KWW (Gurd *et al.*, 2019), whilst FOG, and organic N

content remained constant (Table 1). Removal rates for each of the different organic fractions were normalised to COD units for ease of comparison.

### 3.2.1 Glucose as sole carbohydrate source

Cell dry weights (CDW) for all the glucose concentrations were determined after 8 hours, equivalent to the end of log-phase growth. Mean CDWs were fairly consistent (0.20 – 0.24 g/l) in all series except for 100 mg/l which was only 0.09 g/l (COD:N - 25:1). Cell numbers were lowest in the 1000 mg/l series –  $2.5 \cdot 10^8$  cfu/ml compared to  $3.6 \cdot 10^8$  cfu/ml at 100 mg/l and  $6 \cdot 10^8$  cfu/ml at the higher concentrations. Cell dry weight, therefore, changed from 247 fg/cell in media with 100 mg/l of initial glucose to 359, and 321 fg/cell in media with 2000 and 4000 mg/l of glucose respectively (Figure 2A). Optimum cell weight of  $919 \pm 137$  fg/cell was achieved using 1000 mg/l of glucose. Similar results were reported by Vrede *et al.* (2002), who also found a mean 75% cell weight decrease in C-limited bacterioplankton, and a less dramatic decrease of 35% under N-limitation compared to exponentially growing cells.

The data reported in Figure 2B shows mean observed cell growth yield ( $Y_{Obs}$ ) under glucose limitation in the 100 mg/l data series as 0.76 mg-cell COD/mg-substrate COD (calculated as reported in 2.2). This is a close value to the maximum true yield for growth on carbohydrate predicted at 0.71 mg-cell COD/mg-substrate COD which is achieved through the most efficient use of carbon for growth at the optimum COD:N ratio of 20:1 (Grady *et al.*, 2011). In the current work, carbon use efficiency during log phase growth is described by the relative uptake ratio of theoretical COD:N (calculated from reductions in organic components and total nitrogen over the first 8 hours) which should also be near 20:1 if carbon is used purely to drive growth (optimum efficiency). Figure 2B shows the lowest COD:N uptake was achieved at  $25 (\pm 3):1$  in association with maximum cell yield for the 100 mg/l glucose data set (initial COD:N 35:1). When available glucose concentrations increased above 1000 mg/l (initial COD:N  $\geq 53$ ), bacterial populations responded by removing more organic carbon through increasing carbon catabolism over anabolism, reflected in COD:N uptake increasing to 42 - 50:1, in association with cell yields dropping to 0.23 mg-cell

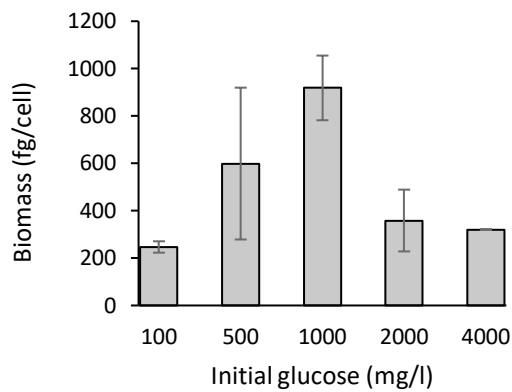
COD/mg-substrate COD. A strong negative correlation ( $R = -0.84$ ) between  $Y_{Obs}$  and COD:N uptake illustrated in Figure 2B indicates that the parameter of COD:N uptake is a useful tool for monitoring bacterial response to available substrate in the absence of direct biomass yield measurements.

Removal rates for each of the different organic fractions in media containing glucose as a source of carbohydrates, are presented in Figures 2C and 2D.

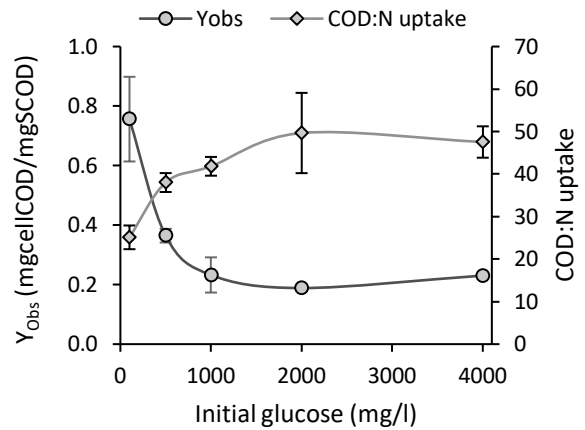
Growth during the log phase was almost exclusively supported by the carbohydrates portion, with minimal contribution from FOG ( $0 - 7.7$  mgCOD/l/h). FOG removal during this phase was limited ( $2-12$  mgCOD/l/h), particularly in association with low bacterial populations in the 100 mg/l series, and by residual glucose availability at high initial COD:N in the 4000 mg/l series.

An increase in FOG removal rate had been expected in the 100, 500 and 1000 mg/l batch reactors after the first 8 hours, once glucose had been completely exhausted, as a response to carbon limitation. However, this was not the case, (Figure 2D). As COD:N uptake during log phase in this study was in excess of the 20:1 ratio for growth requirements, it is likely that the cells had produced more readily bioavailable overflow of metabolites, such as acetate, thereby limiting the need for FOG hydrolysis. In fact, removal time-course data suggests that FOG uptake may have been delayed rather than repressed, in that uptake rates increased during the course of the experiment. For example, maximum removal rates of  $8.7 \pm 0.2$  mg-FOG/l/h ( $24.9 \pm 0.6$  mgCOD/l/h) were observed between 38 and 62 hours in the 1000 mg-glucose/l series (COD:N - 53:1). Whereas, removal remained repressed at the highest concentration, reaching only  $1.2 \pm 0.4$  mg-FOG/l/h. A similar response was reported by Thanh *et al.* (2010) who demonstrated that *B. licheniformis* grown on 4.4 mM glucose ( $\sim 800$  mg/l) secreted around half this amount as acetate and other overflow metabolites into the medium, which were subsequently consumed during stationary phase. In the glucose-limited 100 mg/l series, lower substrate consumption (estimated as  $14 \pm 4$  fg-COD/cfu/h) was insufficient to generate enough maintenance energy to support stationary phase population levels, as suggested by their decline over the course of the experiment (data not shown).

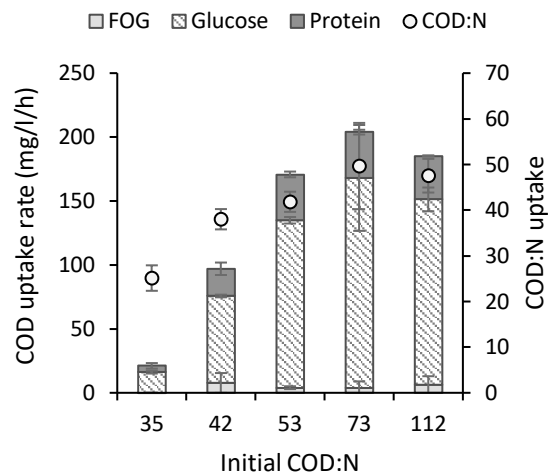
(A) Cell dry weight



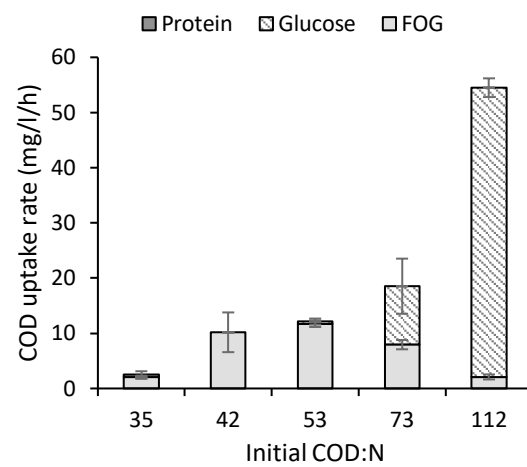
(B) Cell growth yield vs COD:N uptake



(C) Log phase



(D) Stationary phase

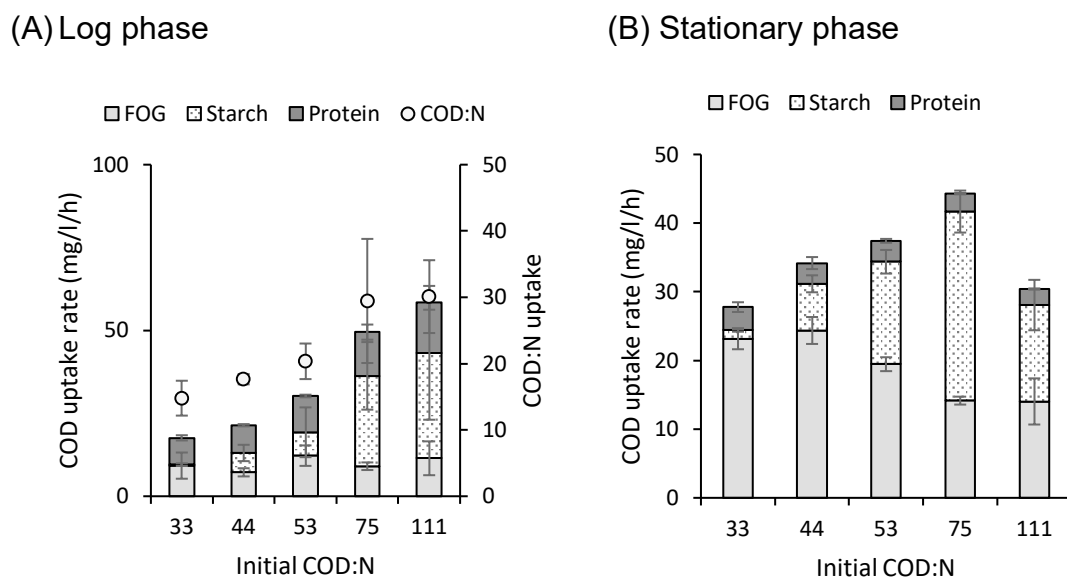


**Figure 2.** Influence of glucose on bacterial FOG removal in synthetic wastewater. (A) bacterial cell dry weight at different concentrations of initial glucose. (B) Comparison of observed growth yields and relative uptake of theoretical COD and N. Theoretical COD uptake rates calculated for individual substrate fractions (bars), together with total COD:N uptake rates (circles) during (C) log phase (0 – 8 hours) and (D) stationary phase (8 – 62 hours) at different initial glucose concentrations.

### 3.2.2 Starch as sole carbohydrate source

Similarly to glucose, starch-based media showed a positive correlation between increasing initial carbohydrate concentrations (and COD:N) and both COD equivalent uptake rates and COD:N uptake. Total log-phase substrate removal rates were considerably lower (18 – 58 mgCOD/l/h) than in the presence of

readily-metabolisable glucose (21 – 204 mgCOD/l/h) (Figure 3A and 2C, respectively). However, growth on the starch media was supported by a more even consumption of FOG, starch and protein (Figure 3A). Carbohydrate uptake rates were considerably lower in starch compared to glucose media: 0.3 – 32 mgCOD/l/h and 16 – 164 mgCOD/l/h, respectively.



**Figure 3.** Influence of starch on FOG removal. Theoretical COD uptake rates calculated for individual substrate fractions (bars), together with total COD:N uptake rates (circles) during (A) log phase (0 – 8 hours) and (B) stationary phase (8 – 62 hours).

In addition, a minimum lag of 4 hours was observed before particulate starch began to be hydrolysed into soluble fragments  $<0.45\mu\text{m}$  (data not shown).

Therefore, it is likely that early growth was predominantly supported by other readily available substrates in the media, such as amino acids. In both conditions, starch and glucose, log-phase growth was largely supported by readily available substrates, it is therefore important to evaluate the metabolism during stationary phase of growth to evaluate the real impact of carbohydrates on FOG degradation.

During stationary phase metabolism, higher FOG uptake rates in the starch media (14 – 24 mgCOD/l/h) were consistent with the production of enzymes

associated with the metabolism of less biodegradable carbon sources in response to readily-available carbon limitation (Egli, 1995). For example, Voigt *et al.* (2007) demonstrated up-regulation in a number of genes associated with metabolism of alternative carbon sources, including amino acids and lipids, when *B. licheniformis* cells were subjected to glucose starvation. Total COD uptake rates were consistently higher in the starch media (28 – 44 mgCOD/l/h) resulting in stable mean stationary phase populations, with a final cell uptake rate of 45 – 78 fg-COD/cfu/h.

Furthermore, starch was never completely exhausted even at the lowest initial concentration: residual concentrations varied from 17 – 69% suggesting that FOG became the preferred substrate once lipolytic enzymes were produced.

Comparison of COD removal rates in Figures 2B and 3B show that carbohydrates had a strong influence on FOG degradation during stationary phase and that the carbohydrates contained in the wastewater will therefore have an impact on the outcomes of FOG bioremediation.

### **3.3 Influence of protein on FOG removal**

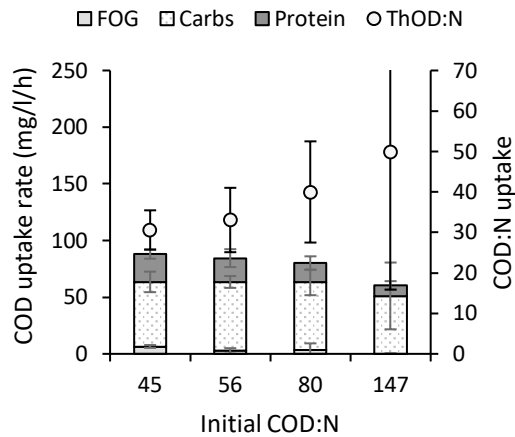
To investigate the importance of the wastewater individual components and initial COD:N ratio, batch experiments content were conducted under the same environmental conditions as those reported in 3.2, but with media comprising of constant carbohydrate concentrations (500 mg/l of glucose and 500 mg/l of starch) and with varying soy protein hydrolysate (SPH) to achieve TN concentrations of 20, 40, 60 and 80 mg/l.

Total COD uptakes rates showed a moderate negative correlation ( $R = -0.67$ ) with initial COD:N, linked to decreasing protein-nitrogen availability (Figure 4C).

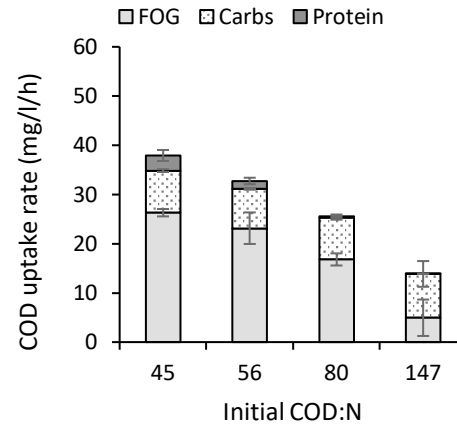
Mean stationary phase FOG uptake rates were higher than at comparable initial COD:N ratios in the starch media. For example 26, 23 and 17 mg/l/h at initial COD:N 45, 56 and 80, compared to 24, 19 and 14 mg/l/h at COD:N 44, 53 and 75 in the starch media. Yet both data sets show a similar negative correlation between FOG uptake and initial COD:N. As initial carbohydrates concentration was constant, the results are a clear indication of the influence of COD:N ratio on FOG removal. Apart from the most N-limited series (COD:N 147), N uptake

continued beyond 8 hours. Whilst continued N uptake in the starch dataset was associated with an approximate doubling in cell numbers between 8 and 16 hours, cell growth in this experiment was not significant.

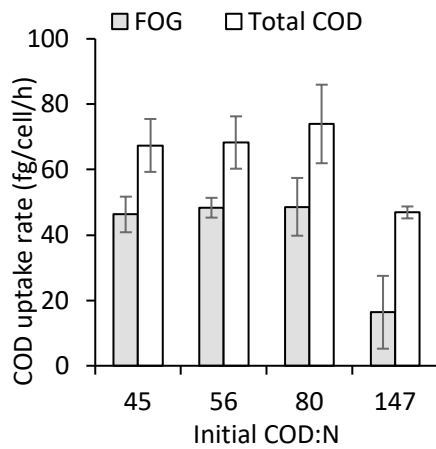
(A) Log phase



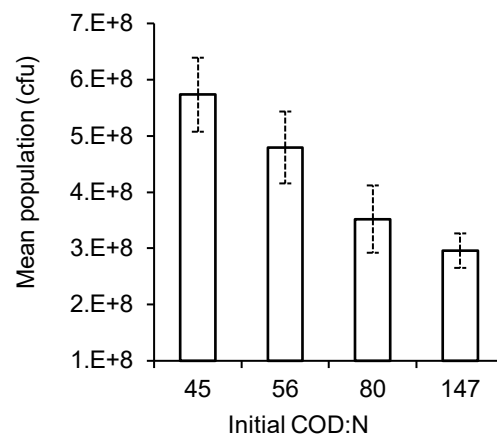
(B) Stationary phase



(C) Cell COD uptake



(D) Stationary phase population



**Figure 4.** Influence of protein on FOG removal. Theoretical COD uptake rates calculated for individual substrate fractions (bars), together with total COD:N uptake rates (circles) during (A) log phase (0 – 8 hours) and (B) stationary phase (8 – 62 hours). (C) COD uptake rates per cell in stationary phase, and (D) mean stationary phase bacterial population concentrations at different initial COD:N ratios.

Mean stationary phase populations were smaller with increasing COD:N (Figure 4D). Thus, although COD removal rates per cell were similar in COD:N 45, 56, 80 cultures (67 – 74 fgCOD/cell/h), increasingly larger populations led to higher



bulk removal (Figure 4C). The highest carbon-excess cultures at COD:N 147 appeared to also demonstrate a cellular response with lower total COD uptake of only 47 fgCOD/cell/h.

### **3.4 Impact of co-substrates on FOG removal in synthetic wastewater**

KWW is typically rich in organic matter and relatively low in ammoniacal nitrogen compared to mixed domestic effluents with the majority of N provided by protein. The optimum nutrient balance for efficient WW treatment is generally assumed to occur at an initial COD:N ratio of 20:1: lower ratios are said to be carbon-limiting leaving residual nitrogen in solution, whilst higher, nitrogen-limiting ratios can be accommodated through partitioning more carbon energy into non-growth associated processes. Optimum criteria were closely met at the lowest concentration in the glucose media experiment (initial COD:N 35), wherein near maximum growth yields were achieved in association with COD:N removal of  $25 \pm 3$ . With increasing concentration, more COD energy was dissipated, -resulting in COD:N uptake up to a maximum of 50:1 and lower cell yields. The same response was observed in the other experiments with starch and mixed carbohydrate compositions.

First order FOG removal rate constants ( $k$ ), were calculated for all substrate conditions for the 16 – 62 hour time period (Figure 5). All data sets showed similar negative correlation between FOG removal and initial COD:N such that FOG uptake was favoured by more nitrogen-rich environments. FOG removal was consistently lowest in the glucose media wherein it was likely that stationary-phase maintenance energy was supplied by overflow metabolites excreted during log phase, thus delaying the need for FOG hydrolysis. Despite an initial COD:N of 35 in the 100 mg-glucose/l cultures, bacterial growth was poor due to limitation of readily-available carbon, leading to low FOG  $k$  values ( $0.001 \text{ h}^{-1}$ ) during stationary phase.

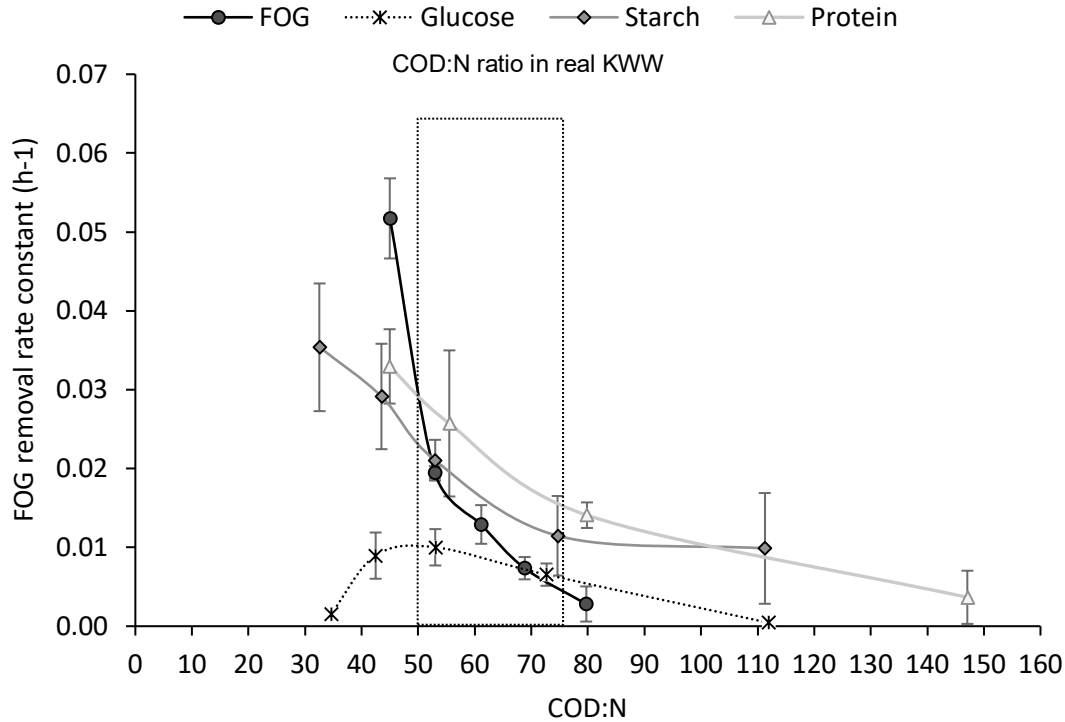
FOG  $k$  was up to an order of magnitude higher when glucose was replaced by starch. Maximum starch removal occurred in early stationary phase followed by a switch to FOG as the preferred substrate after 16 hours. As COD:N ratio should

have little influence on COD uptake in stationary phase, we suggest that residual N availability has an influence on enzyme production which also requires a source of nitrogen. TN was removed more slowly in the starch media during log phase (1.2 – 1.9 mg/l/h) than in glucose media (2.6 – 4.1 mg/l/h excluding the 100 mg/l series), leaving more nitrogen in solution. Nitrogen uptake continued throughout stationary phase whilst populations declined from a maximum at 16 hours, suggesting that N was used for enzyme synthesis allowing continued hydrolysis of starch and FOG. Similarly, N uptake during early stationary phase in the protein variation media was not associated with significant bacterial growth but was associated with increasing enzyme activity (Supplementary data. Figure 3S).

It appears that microbial response is governed by the ratio of readily-available carbon and nitrogen according to the model proposed by Schimel & Weintraub (2003) developed for microbial decomposition in soils, where microorganisms are commonly limited by readily-available carbon. Those authors propose that enzyme synthesis is prioritised and biomass production will only ensue when the carbon and energy requirements for more enzyme synthesis and cellular maintenance are met. Given that FOG contains more electrons than starch as reflected in their relative COD factors (2.88 and 1.07 respectively), and thus delivers more carbon and energy by mass, it is likely that bacteria in our systems, would favour the synthesis of lipase(s) rather than starch-degrading enzymes. This would also explain the relatively higher rates of FOG uptake in stationary phase when both fractions remained.

A marked difference in carbon uptake was observed in media with easily biodegradable carbohydrate (glucose) compared to more slowly-hydrolysable starch. Nitrogen was not completely exhausted under any conditions, and thus would appear not to be a limiting factor (data shown in figure 2S). Only  $56 \pm 4\%$  of initial N concentrations were removed, which is consistent with the proportion of free amino acids in the soybean protein hydrolysate used for the media (Sheffield Bioscience, 2014). This indicates that the remaining 40% of nitrogen, available in the form of peptides, was less accessible for microbial uptake and anabolism. These results suggest that, biodegradability of both organic carbon

and nitrogen should be considered when evaluating microbial response to COD:N.

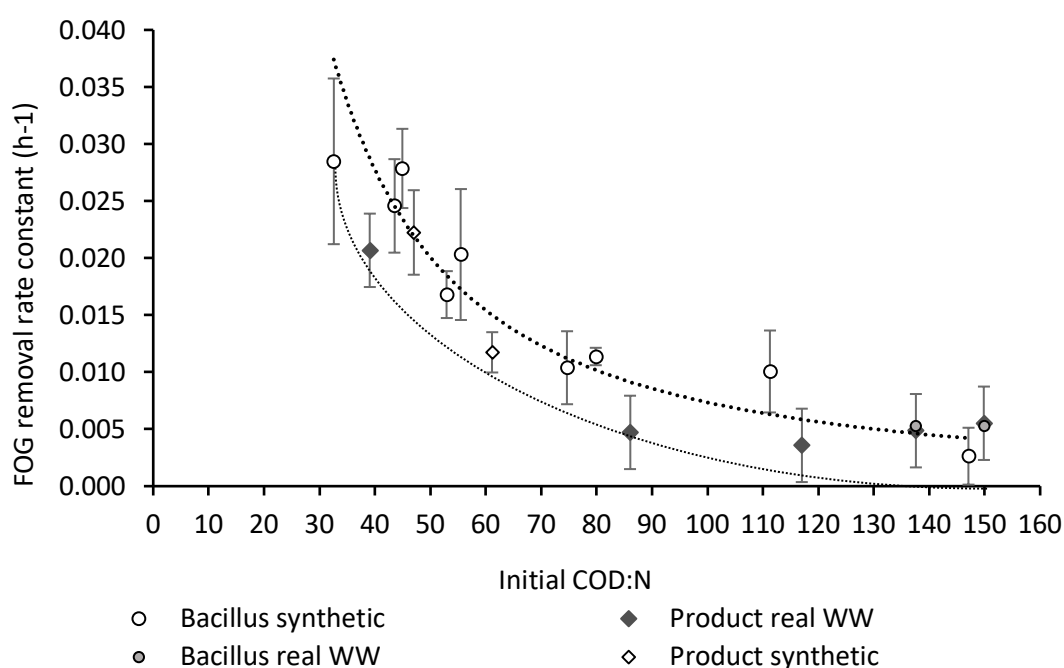


**Figure 5.** First order FOG removal rate constants (16 – 62 hours) in synthetic media with varying concentrations of either FOG, glucose, starch or protein resulting in a range of initial COD:N

### 3.5 Bioadditions performance in real kitchen wastewater

To validate our results, degradation experiments were conducted using real kitchen wastewaters (from a food service establishment on campus, with composition comparable to that reported previously in Gurd *et al.* 2019) dosed with either *Bacillus licheniformis* or a commercial bioadditive product. Variability was observed in the FOG removal profiles which was likely due to difficulties in achieving an initial homogenous distribution of FOG between sub samples taken from the bulk sample. FOG removal rate constants ( $k$ ) were calculated for the whole experimental period (0 – 62 hours) to normalise the unavoidable variations of real KWW. Initial theoretical COD:N ratios were estimated using COD conversion factors for FOG, carbohydrate and organic nitrogen measurements

as reported in 2.5, and were further varied by addition of starch or ammonium chloride (See Table 2S in supplementary material for details of substrate composition). FOG removal response for the commercial bioadditive in real WW showed a negative relationship with initial COD:N, following a parallel trend line to experiments with *Bacillus* in synthetic WW (Figure 6). FOG  $k$  was lowest towards COD:N  $\geq 117:1$  for both the commercial product and *Bacillus* in real WW, and were not significantly different to results in synthetic media at COD:N 147,



**Figure 6.** FOG removal rate constants for *B. licheniformis* and a commercial bioadditive product in real and synthetic wastewaters with varying COD:N ratios.

Furthermore, FOG  $k$  for commercial product cultures in the test protocol synthetic composition lay on the real WW trend line at COD:N 61, and the value of 0.0222  $\text{h}^{-1}$  at COD:N 47 was also not significantly different from real WW - 0.0207  $\text{h}^{-1}$  at COD:N 39.

Using synthetic and real wastewaters of varying compositions, a clear negative relationship was determined between FOG removal and initial COD:N over a range of 33:1 to 150:1. FOG removal rate constants were comparable in synthetic and real WW with similar COD:N for cultures of *Bacillus licheniformis* NCIMB 9375, and a commercial bioadditive product. Results are reported in Figure 6. Overall the data suggest that FOG removal in our synthetic media and test

bacteria, is representative of that achieved in real kitchen wastewaters of varying compositions: the dominant influence being the initial ratio of COD:N.

### **3.6 Implications for biological remediation**

In addition to high concentrations of fat, oil and grease (FOG), wastewater from food service establishments contains high quantities of carbohydrates and proteins, which could also contribute towards blockages in sewer networks. As previously reported, COD:N ratios in FSE WW can vary from 30:1 to 150:1 (Gurd *et al.* 2019). These values are considerably higher than the reported optimum ratio of 20:1 for wastewater degradation. Carbohydrates and organic nitrogen are also known to impact on the ability of microorganisms to degrade complex organics, such as FOG. This can have obvious implications for their biological remediation of kitchen wastewater and hence FOG deposition in the sewage system. Our results has demonstrated the importance of COD:N on FOG removal. COD:N ratio was the dominant influence on FOG removal in both synthetic and real WW. The traditional accepted ratio for successful biological treatment is 20:1 – the optimum balance between carbon energy and nitrogen to produce new biomass. However, this work showed that populations could respond to carbon excess by altering their metabolic processes to use carbon less efficiently resulting in relative COD and N uptake of up to 50:1 – well in excess of growth requirements. Notwithstanding, barely any FOG COD was removed in association with bacterial growth with populations showing a preference for easily-metabolisable organic matter such as glucose or free amino acids. Instead FOG removal was initiated after around 14-16 hours as populations reached the stationary phase. Higher FOG removal rates were observed in media with lower initial COD:N ratios where residual nitrogen remained available beyond log-phase for the production of FOG-degrading hydrolytic enzymes during stationary phase which allowed for continued FOG removal to support cell maintenance.

This study highlights the importance of KWW characterisation. Although WW composition is highly variable day to day, water composition (COD:N:P) and FOG concentrations should be tested before starting to use bioadditives. This will help

to identify emissions from specific premises and adjust their composition accordingly. For example, adding nitrogen compounds to mitigate carbon excess conditions in kitchen WW can enhance microbial FOG degrading ability. However, as COD:N ratios below 20:1 can also impact negatively on treatment, it is important to gauge likely site-specific ranges when devising bespoke FOG management solutions to prevent over-dosing of nutrients.

The suppression effect of glucose on FOG degradation at any concentration tested suggests that bioadditive treatment may not be an appropriate option for wastewaters from sugar-based industrial manufacturing, whilst low-sugar, starch-based compositions may be more appropriate.

Another important implication of this work is related to bioremediation requirements for contact time. The present work identified that FOG removal does not begin until stationary phase under any of the substrate conditions tested, with a lag time of at least 14 - 16 hours. This suggests that there may be a requirement for a pre-culturing phase prior to dosing in a separator/trap or on-line in sewers to initiate lipase production. Indeed, some bioadditive systems are designed to administer the product following pre-culturing in a separate tank. However, the ability of bacteria to form biofilms on waste pipe walls also allows them to exist as 'pre-activated' populations in a variety of physiological states including stationary-phase state (Rani et al., 2007). Biofilms initially form because many bacteria have hydrophobic cell surfaces and thus they are attracted to surfaces by electrostatic interactions (Arnaouteli et al., 2016). FOGs are also hydrophobic and tend to adhere to the pipe walls. This brings FOG in direct contact with the biofilm layer thereby increasing contact time indefinitely provided they can be degraded at less than, or the same rate, as deposition.

In this work, highest FOG removal rates for *B. licheniformis* of 8 – 9 mg/l/h were observed in media with different compositions mixed at COD:N ratios of 56:1 and 45:1 respectively, and at  $9.5 \pm 0.4$  mg/l/h at COD:N 42:1. FOG degradation rates needs to be at least equal to adherence rates on a biofilm surface to prevent net accumulation of deposit. Although this was beyond the scope of this work it is assumed that these conditions will be met during periods of inactivity in the kitchen, such as overnight.

Investigations into microbial removal of organic matter in sewer environments has found biofilms show a high degree of metabolic activity and extracellular enzymes activity, with cell counts in the biofilm layer higher than in those in activated sludge wastewater treatment plants (Chen et al., 2003; Lemmer et al., 1994, Hassard et al. 2018). Moreover, (Chen and Leung, 2000) determined that oxygen uptake rates and ATP contents were twice as higher in sewer sediments than in the sewage phase indicating that biofilm populations levels and microbial activity were double those in the wastewater phase. By inference, FOG removal rates in drain pipes might be three times higher than those observed in the aqueous phase in this work.

## **4 Conclusions**

Kitchen wastewater composition had a strong influence on bioremediation outcomes, explaining why laboratory and full-scale studies on bioadditions often have inconsistent results. COD:N was evaluated over a wide range of values, from traditionally accepted optimum conditions (20:1), up to 150:1, to represent more challenging conditions. A strong negative correlation was identified between bacterial cell yields and COD:N uptake. The current work has also demonstrated the importance of the COD:N on FOG removal. Higher FOG removal rates were observed in media with lower COD:N. For example, uptake rates per cell were similar over a COD:N range 45 – 80 but population levels decreased which translated into lower overall FOG uptake. At highest tested ratio (147) there was a 3-fold decrease in per cell uptake, together with smaller populations overall FOG uptake 5 times lower than at COD:N 45. Finally, FOG removal was suppressed in the presence of glucose, by low bacterial populations at low glucose concentrations, and residual glucose at higher concentrations, but increased with increasing organic nitrogen content. Although WW composition is highly variable day to day, water composition (COD:N) and FOG concentrations should therefore be tested before starting to use bioadditives to limit process failure.

## 5 Acknowledgments

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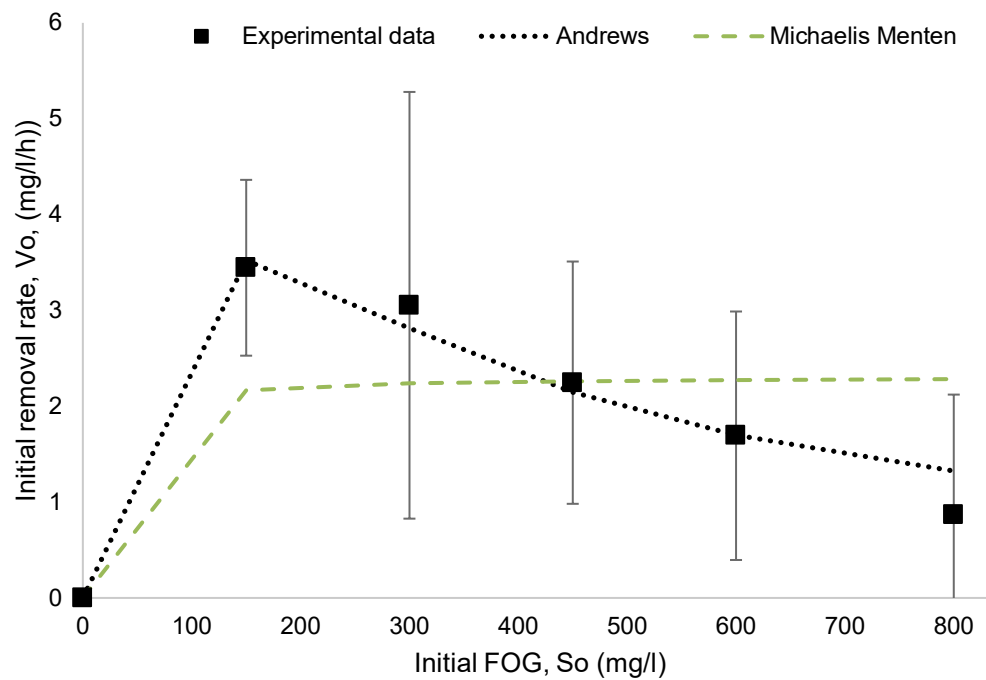
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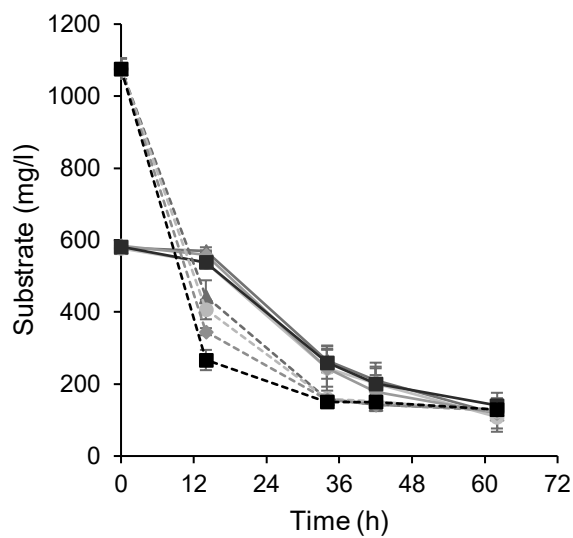
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## Supplementary data

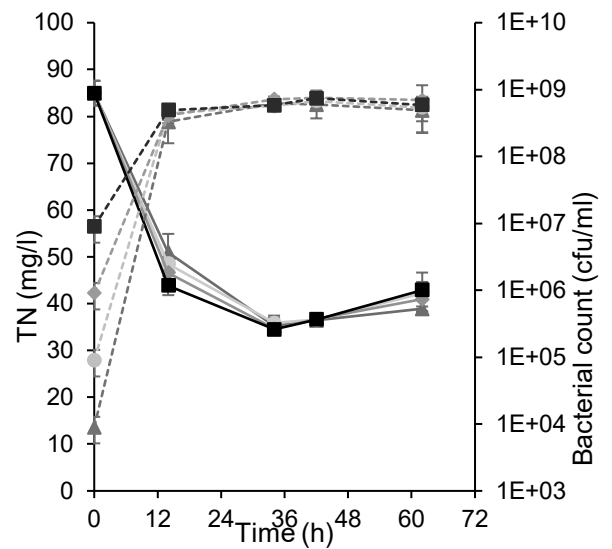


**Figure 1S.** FOG removal rate data fitted to the Michaelis Menten, and Andrew's substrate inhibition enzyme kinetic models calculated using 'Solver' in Microsoft Excel. Data are presented as means of triplicate independent experiments with error bars as standard deviation.

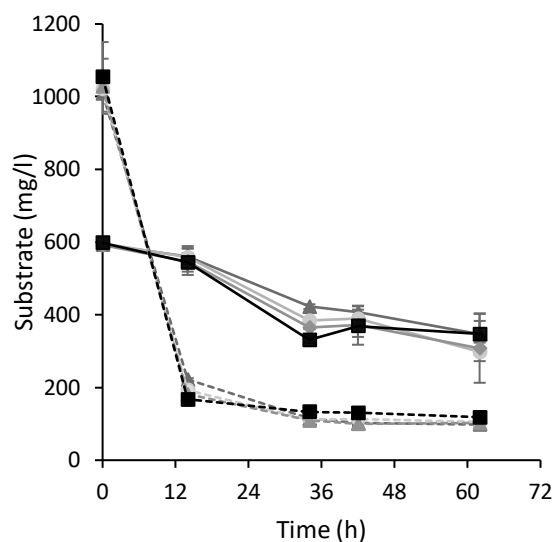
(A) Reference bacteria: FOG and carbohydrates



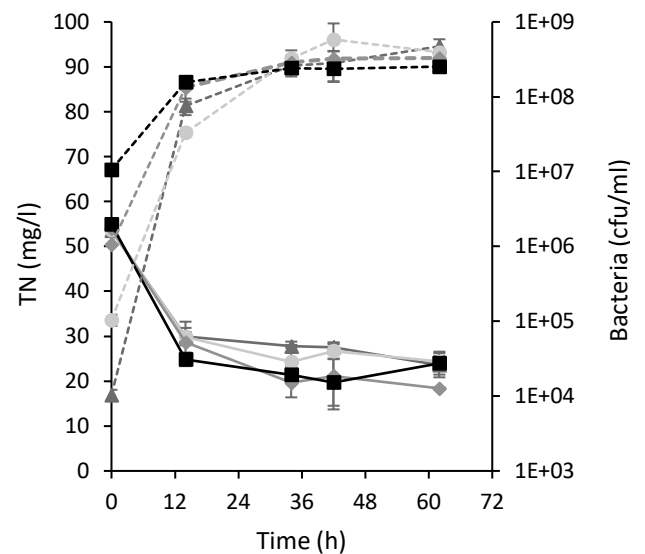
(B) Reference bacteria: TN and growth



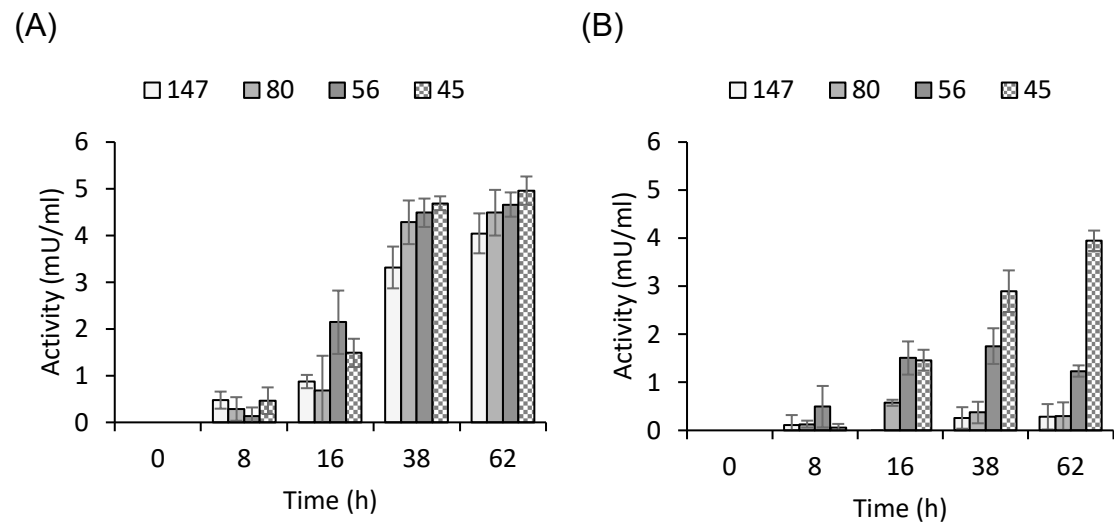
(C) Commercial product FOG and carbohydrates



(D) Commercial product TN and growth



**Figure 2S.** Substrate removal profiles *B. licheniformis* (A and B) and a commercial product (C and D) in synthetic wastewater (FOG 600 mg/l, carbohydrates 500 mg/l glucose and 500 mg/l starch, soy protein hydrolysate 400 mg/l) at different initial doses (▲ 1E+04; ● 1E+05; ◆ 1E+06; ■ 1E+07): (A and C) FOG removal (solid lines) and carbohydrate removal (dashed lines); (B and D) bacterial growth (dashed lines) and total nitrogen variation (solid lines).



**Figure 3S.** Influence of varying protein concentration on FOG removal: lipase activity over time for (a) butyrate-specific enzyme and (b) octanoate-specific enzyme.

**Table 1S.** Base media composition for synthetic wastewater, containing trace elements and surfactants in mean concentrations identified in real FSE wastewater (Gurd et al. 2019).

Component	Concentration (mg/l)
Calcium chloride dihydrate	100
Potassium chloride	70
Magnesium sulphate heptahydrate	40
Ammonium chloride	10
Iron chloride	0.27
Zinc sulphate heptahydrate	0.16
Copper sulphate	0.07
Manganese sulphate monohydrate	0.045
Cobalt nitrate hexahydrate	0.002
Ammonium heptamolybdate tetrahydrate	0.001
Triton X-100 (non-ionic surfactant)	14
<b>Added post autoclave:</b>	
Sodium dodecyl sulphate (anionic surfactant)	30
Sodium phosphate buffer	30 mM
Vegetable oil (rapeseed) (Tesco)	150-800
Soy protein hydrolysate (Amisoy)	150-600
Cornflour – 88% starch (Tesco)	100-4000
Glucose	100-4000

**Table 2S.** Initial concentrations of FOG, carbohydrate and nitrogen in real wastewater used in the degradation trials at different initial COD:N.

FOG (mg/l)	Carbohydrate (mg/l)	TN (mg/l)	COD:N
698 ± 61	222 ± 11	16 ± 1	150
1141 ± 77	883 ± 16	33 ± 1	138
356 ± 78	3028 ± 236	56 ± 3	86
356 ± 78	3028 ± 236	*122 ± 6	39
356 ± 78	#4654 ± 186	56 ± 3	117

\*Amended with additional ammonium chloride. #Amended with additional starch